

Exopolysaccharide Productivity and Biofilm Phenotype on Oral Commensal Bacteria as Pathogenesis of Chronic Periodontitis

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1. Introduction

Exopolysaccharide (EPS) productivities in many bacteria have been associated with pathogenicity in mammalian hosts as providing extracellular matrices to form biofilm (Costerton et al., 1995). Bacteria assuming biofilm-forming capacity have enormous advantages in establishing persistent infections (Costerton et al., 1999). Chronic periodontitis is caused by dental plaque known as a complex biofilm which consists of several hundred different species of bacteria (Chen, 2001; Socransky and Haffajee, 2002; Lovegrove, 2004). While sucrose-derived homopolysaccharides are well known substrates which mediate adhesion of bacteria to the tooth surface and co-aggregation interactions between species of oral bacteria in the dental plaque (Russell, 2009), recent studies suggest that each bacterium produces distinctive EPS components in a sucrose-independent manner and can form so called single species biofilm (Branda et al., 2005). In the oral cavity, several species of oral bacteria are known to produce their own EPS with this manner (Okuda et al., 1987; Dyer and Bolton, 1985; Kaplan et al., 2004; Yamane et al., 2005; Yamanaka et al., 2009; Yamanaka et al., 2010). In this chapter, we will describe a possibility that a single species biofilm in the oral cavity can cause persistent chronic periodontitis along with the importance of dental plaque formation and maturation with sucrose-derived polysaccharides.

2. Dental plaque formation with sucrose-derived polysaccharides

Dental plaque is defined as a community of oral bacteria on a tooth surface in which microorganisms are found embedded in EPS and intimately communicate each other via several different communication pathways such as auto-/co-aggregation, metabolic communication, quorum sensing and competent stimulation peptides (Rickard et al., 2008). A recent study using pyrosequencing technique showed that dental plaque harbors nearly 7000 species-level phylotypes (Keijser et al., 2008). Therefore, dental plaque is described as

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mix-/multi-species biofilm as well. A widely accepted theory of dental plaque formation is an organized sequence of events (Marsh, 2004). 1) The enamel surface of tooth is covered by acquired pellicle which consists of salivary proteins. 2) Initial colonizers of oral bacteria adhere on the tooth surface via physico-chemical interactions between the bacterial cell surface and the pellicle matrices, and then establish firmer adhesin-receptor mediated attachment. A study (Nyvad and Kilian, 1987) using cultivation technique showed that the initial colonizers are predominated by streptococci such as *Streptococcus sanguinis*, *Streptococcus oralis* and *Streptococcus mitis*. Gram-positive rod *Actinomyces* spp, *veillonellae*, and *Rothia mucilaginosa* were frequently found in the early stage of plaque formation (Nyvad and Kilian, 1987). After the colonization of these pioneers, bacteria that have glucosyltransferase (GTF) or fructosyltransferase (FTF) start to provide sucrose-derived EPS as plaque substrates (Russell, 2009). The EPS can be soluble or insoluble and the latter make a major contribution to the structural integrity of dental plaque and can consolidate the attachment of bacteria in dental plaque. Among previously known initial colonizers, *S. sanguinis* can provide water-soluble/insoluble EPSSs because this organism possesses both GTF and FTF. In this environmental niche, co-adhesion between initial colonizers and secondary colonizers occurs. 4) Then, more secondary species adhere to the developing dental plaque resulting in the increased number of bacteria through the continued integration and cell divisions (Rickard et al., 2008). 5) When dental plaque as multi-species biofilm has developed and become matured, the flora gradually changes from Gram-positive cocci and *Actinomyces* to the one containing certain amount of Gram-negative organisms (Chen, 2001; Herrera et al., 2008; Paster et al., 2001; Socransky et al., 1998). The change in dental plaque flora is also associated with the extension of the plaque subgingivally, and it is evidently shown that this phenomenon causes the plaque-associated complex symptoms in periodontal tissues (Darby and Curtis, 2001; Dahmen, 1993). This theory well explains the dental plaque formation, maturation and the plaque-associated complex in modern day since the production and consumption of sucrose increased dramatically in nineteenth century. However, considering the facts that ancient specimens showed carious lesions localized on the root surfaces and simultaneous absence of coronal lesions, oral microorganisms might have a strategy in sucrose-independent manner to form dental plaque on the tooth surface around the gingival crevice. The periodontal bone loss is also found on the ancient specimens (Meller et al., 2009; Gerloni et al., 2009). Therefore, it is conceivable that the dental plaque developed in sucrose-independent manner could be pathogenic for periodontal tissues and can cause chronic periodontitis lesions.

2.1 Initial colonizers on the tooth surface and their capacity to form biofilm

More recent studies using molecular methods and a retrievable enamel chip model have revealed a new line-up of initial colonizers though the early dental plaque microflora varies at subject-specific basis (Diaz et al., 2006; Kolenbrander et al., 2005). In initial plaque on the chip at four to eight hours, *Streptococcus* spp. was dominant while *Veillonella*, *Gemella*, *Prevotella*, *Niesseria*, *Actinomyces* and *Rothia* were also frequently found. Among streptococci, *S. oralis*, *S. mitis*, *S. infantis*, *S. sanguinis*, *S. parasanguinis*, *S. gordonii*, *S. cristatus* and *S. bovis* were found in the early dental plaque. Although this bacterial community can be given substrates by bacteria which synthesize EPS in sucrose-dependent manner, we recently found that several bacteria newly nominated as initial colonizers have the ability to produce their own EPS in sucrose-independent manner and to form biofilms.

The presence of dense meshwork structures under scanning electron microscopy (SEM) is a typical feature for biofilm forming organisms. The appearances of *Escherichia hermannii* (Yamanaka et al., 2010) with or without EPS production in SEM observation are shown in Figure 1. *E. hermannii* YS-11 isolated from persistent apical periodontitis lesions produced EPS and exhibited cell surface meshwork structures (Fig. 1A). The meshwork structures of *E. hermannii* YS-11 disappeared when *wzt*, one of the ABC-transporter genes, was disrupted by transposon random insertion mutagenesis (Fig. 1B). Complementation of this gene to the transposant restored and dramatically augmented the formation of meshwork structures (Fig. 1, C and D). Such phenotypes are similar to those of *Pseudomonas aeruginosa*, a prototype of biofilm-forming bacteria (Kobayashi, 1995; Yasuda et al., 1999), *Escherichia coli* (Prigent-Combaret et al., 2000; Uhlich et al., 2006), *Salmonella* (Anriany et al., 2001; Jain and Chen, 2006), and *Vibrio cholerae* (Wai et al., 1998).

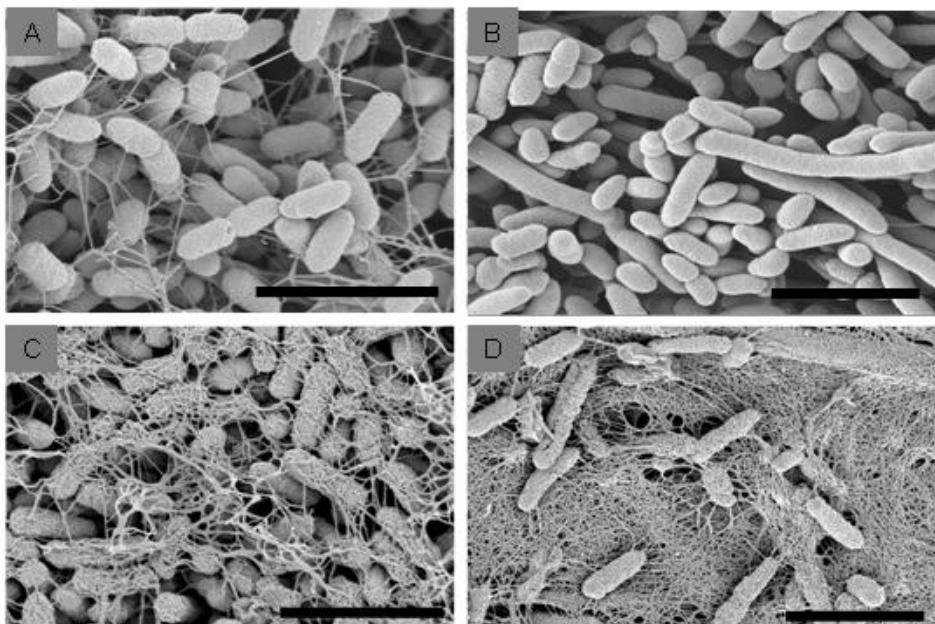


Fig. 1. Scanning electron micrographs showing surface structures of *Escherichia hermannii* strain YS-11 (A; wild type), strain 455 (B; *wzt* transposant) and strain 455-LM (strain 455 with pWZT; C: without IPTG induction; D: with IPTG induction). Bars = 3 μ m

When we observed the surface structures of isolates from saliva of healthy volunteers or from chronic peripheral periodontitis lesions by SEM, similar cell surface-associated meshwork-like structures were observed on *Neisseria*, *S. parasanguinis*, *S. mitis*, *Rothia dentocariosa*, *Rothia mucilaginosa* (Yamane et al., 2010), *Prevotella intermedia* (Yamanaka et al., 2009), *Prevotella nigrescens* (Yamane et al., 2005) and *Actinomyces oris* (Fig. 2). We have investigated the clinical isolates of *P. intermedia* and *P. nigrescens* with meshwork structures and found that the organisms can produce their own unique EPS in sucrose-independent manner (see below). However, it is still unclear whether other initial colonizers possess the

meshwork structures with the same manner. It is important to note that similar tubule-like structures are formed by bacterial nanotubes (Dubey and Ben-Yehuda, 2011) or amyloids (Dueholm et al., 2010).

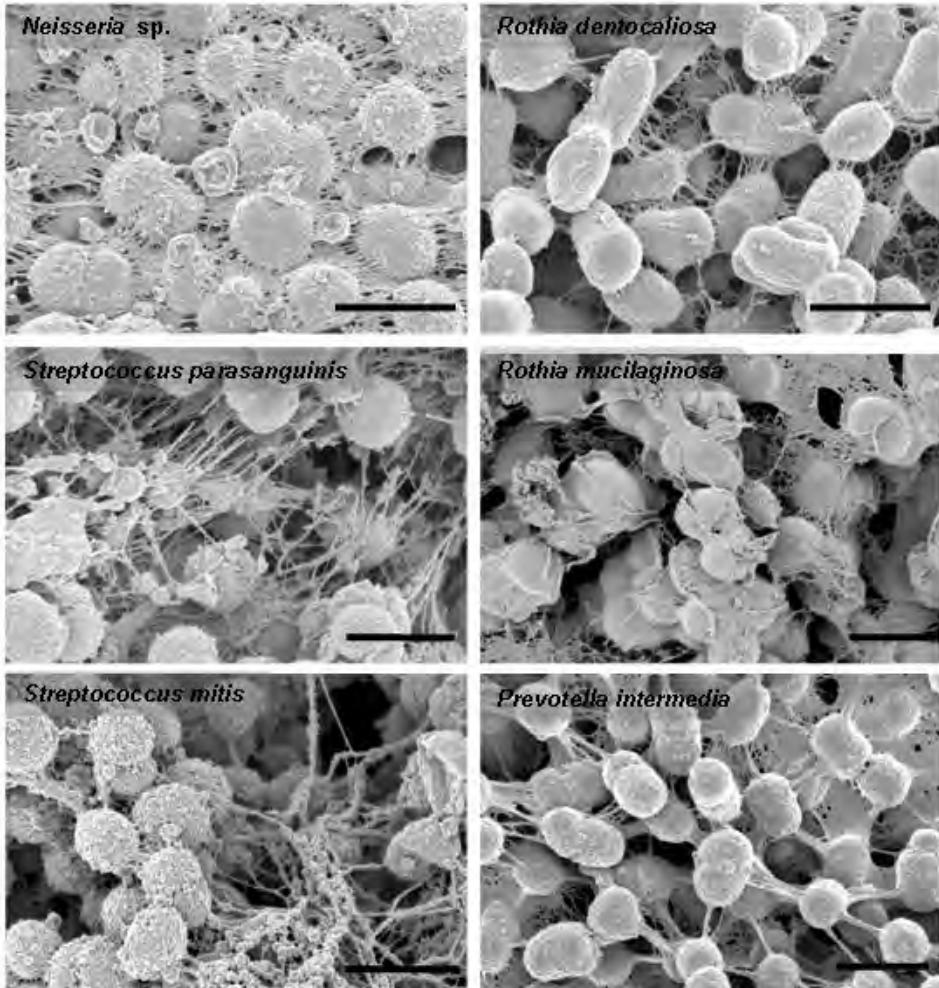


Fig. 2. Scanning electron micrographs showing cell surface structures of oral bacteria known as initial colonizers. A colony of each clinical isolate was used for SEM observation and identification by 16S rRNA gene sequencing. Bars = 2 μ m.

2.1.1 Single species biofilm with unique EPS production on the outside of oral cavity

Practically all bacteria living in their own environmental niche have the capacity to form biofilm by a self-synthesized matrix that holds the cells together and tightly attaches the bacterial cells to the underlying surface. Polysaccharide is a major component of the matrix

in most bacterial biofilms although recent studies have shown that constituents of biofilm matrix vary and that extracellular nucleic acids (Wu and Xi, 2009) or secreted proteins (Latasa et al., 2006) are also used as the matrix. Recent investigations have revealed that each biofilm-forming bacterium produces distinctive EPS components *e.g.* alginate and/or Psl found in *P. aeruginosa* (Ryder et al., 2007), acidic polysaccharide of *Burkholderia cepacia* (Cerantola et al., 1999), collanic acid, poly- β -1,6-GlcNAc (PGA) or cellulose found in *E. coli* (Junkins and Doyle, 1992) (Wang et al., 2004; Danese et al., 2000), cellulose of *Salmonella* (Solano et al., 2002; Zogaj et al., 2001), amorphous EPS containing *N*-acetylglucosamine (GlcNAc), α -mannose, 6-deoxy- α -galactose and α -galactose of *V. cholerae* (Wai et al., 1998; Yildiz and Visick, 2009), polysaccharide intercellular adhesin (PIA) of *Staphylococcus* (Rupp et al., 1999), and glucose and mannose rich components found in *Bacillus subtilis* biofilm (Hamon and Lazazzera, 2001; Ren et al., 2004; Yamane et al., 2009). An enteric pathogen *Campylobacter jejuni* produces EPS that reacts with calcofular white, indicating the polysaccharide harbors β 1-3 and/or β 1-4 linkages. The production of this EPS is considered to be involved in the stress response of this organism together with its surface-associated lipooligosaccharide and capsular polysaccharides (McLennan et al., 2008). Persistent infections caused by biofilm-forming bacteria have been abundantly reported, however, understanding the molecular basis for the synthesis of biofilm matrices is still limited. The bacteria assuming the ability to produce their own polysaccharides and causing infectious diseases (biofilm infections) are listed in Table 1.

EPS-producing bacteria	Constituents of EPS	Biofilm infection
<i>Pseudomonas aeruginosa</i>	Alginate, Psl (mannose- and galactose-rich polysaccharide) or Pel (glucose rich polysaccharide)	Cystic fibrosis pneumonia, contact lenses infection, central venous catheter infections
<i>Burkholderia cepacia</i>	Acidic branched heptasaccharide	Cystic fibrosis pneumonia (cepacia syndrome)
<i>Escherichia coli</i>	Cellulose, colonic acid or poly- β -1,6-GlcNAc (PGA)	Intestinal disorders, urinary tract infections, urinary catheter infections
<i>Vibrio cholerae</i>	Glucose- and galactose-rich polysaccharide	Cholera, diarrheal diseases (the EPS protects this organism from environmental stress)
<i>Salmonella enterica</i> serovar <i>Typhimurium</i>	Cellulose	Gastroenteritis
<i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i>	Staphylococcal polysaccharide intercellular adhesion (PIA)	Endocarditis, central venous catheter infections, urinary catheter infections
<i>Bacillus subtilis</i>	Glucose- and mannose-rich polysaccharide	Opportunistic infections, apical periodontitis
<i>Campylobacter jejuni</i>	EPS contains β 1-3 and/or β 1-4 linkages	Bacterial gastroenteritis

Table 1. EPS-producing bacteria on the outside of oral cavity, constituents of EPS and related diseases.

Oral streptococci such as anginosus group, mitis-group and salivarius-group and *Rothia* are known to cause biofilm infections on prosthetic heart valves and artificial voice prostheses (Donlan, 2001). Interestingly, some clinical isolates of *Streptococcus intermedius* and *Streptococcus salivarius* exhibit dense meshwork structures around their cells suggesting these organisms can form single species biofilm on medical devices though we still do not know the constituents of the matrices (Matsumoto-Mashimo et al., 2008) (Fig. 3).

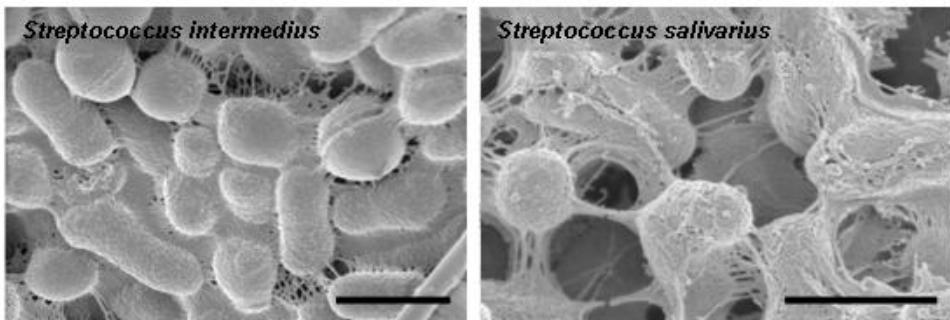


Fig. 3. Scanning electron micrographs showing cell surface structures of clinically isolated *S. intermedius* and *S. salivarius*. Bars = 2 μ m.

2.1.2 Biofilm-forming bacteria from chronic periodontitis lesions and the chemical composition of their EPS

As described above, several periodontopathic bacteria are known to produce EPS or capsular polysaccharides. The production of mannose-rich polysaccharide by *Capnocytophaga ochracea* has been reported (Dyer and Bolton, 1985). The mannose-rich EPS provides this organism with a protection from attack by the human innate immune system (Bolton et al., 1983). Kaplan et al. (2004) reported that *Aggregatibacter actinomycetemcomitans* has a gene cluster which is homologous to *E. coli pgaABCD* and encodes the production of poly- β -1,6-GlcNAc (PGA) (Wang et al., 2004). We found that *P. intermedia* strain 17 produced a large amount of EPS, with mannose constituting more than 80% of the polysaccharides (Yamanaka et al., 2009). The growth of strain 17 was slower than that of *P. intermedia* ATCC 25611 (a reference strain for *P. intermedia*). Viscosity of spent culture media of strain 17 was higher than that of ATCC 25611. Transmission electron microscopy of negatively stained purified EPS showed fine fibrous structures that are formed in bundles. Meshwork structures were represented on latex beads coated with the purified EPS (Fig. 4).

We have also reported that a clinical isolate of *P. nigrescens* can produce a copious amount of EPS consisting of mannose (88%), glucose (4.3%), fructose (2.7%), galactose (2.1%), arabinose (1%) and small amounts of xylose, rhamnose and ribose. Methylation analysis suggested that the EPS is composed of highly branched (1-2)-linked mannose residues (Yamane et al., 2005). Okuda et al. (1987) reported that *P. intermedia* 25611, *Porphyromonas gingivalis* 381 and *P. gingivalis* ATCC 33277 had capsular structures around the cells and that the capsular polysaccharides extracted from *P. gingivalis* 381 contained galactose and glucose as their major constituents. *P. gingivalis* W83 is known to produce capsular polysaccharides, and the

genetic locus for capsule biosynthesis has been identified (Aduse-Opoku et al., 2006). However, these reference strains in our laboratory do not produce capsular polysaccharide or EPS. One possibility is that the tested strains had lost their ability to produce capsular polysaccharides or EPS because of multiple *in vitro* passages of the organisms in the laboratory. Although the molecular basis for biofilm formation in *Rothia* still needs to be elucidated, Yamane et al. (2010) determined the whole genome sequence of *R. mucilaginosa* DY-18, a clinical isolate from persistent apical periodontitis lesions with an ability to produce EPS and exhibit cell surface meshwork structures.

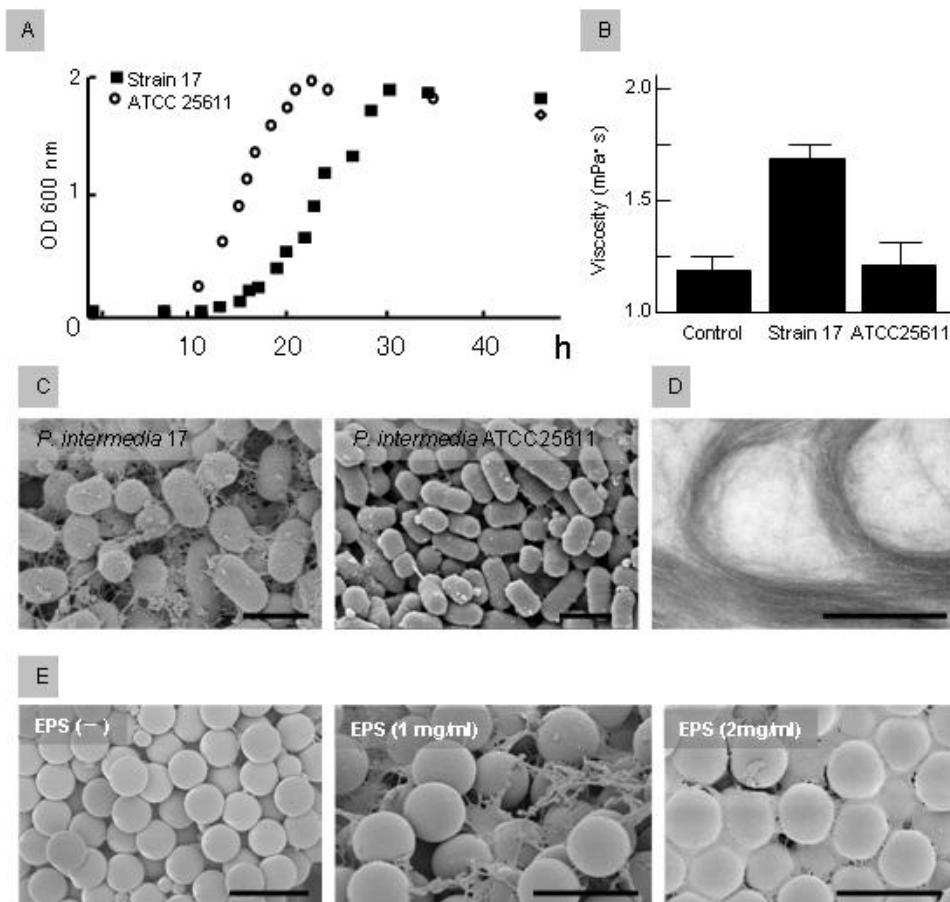


Fig. 4. Comparison of growth (A), viscosity of spent culture media (B) and phenotype between *P. intermedia* strain 17 and ATCC 25611. Bars in C = 1 μ m. Transmission electron micrograph of negatively stained purified EPS from *P. intermedia* 17 cultures (D). Bar = 500 nm. Meshwork structures represented on EPS-coated latex beads (2 μ m in diameter)(E). Bars = 5 μ m.

2.1.3 EPS productivity and biofilm phenotype as virulence factors

It is evidently shown that the slime/EPS production is critical for bacteria to exhibit the resistance to the neutrophil phagocytosis, though some EPS are not essential to bacterial adherence to host cells or for systemic virulence. Jesaitis et al. (2003) demonstrated that human neutrophils that settled on *P. aeruginosa* biofilms became phagocytically engorged, partially degranulated, and engulfed planktonic bacteria released from the biofilms. Deighton et al. (1996) compared the virulence of slime-positive *Staphylococcus epidermidis* with that of slime-negative strain in a mouse model of subcutaneous infection and showed that biofilm-positive strains produced significantly more abscesses that persisted longer than biofilm-negative strains. Our previous studies showed that *P. nigrescens* as well as *P. intermedia* with mannose-rich EPS showed stronger ability to induce abscesses in mice than those of a naturally occurring variant or chemically-induced mutant that lack the ability to produce EPS. TEM observations revealed that test strains with mannose-rich EPS appeared to be recognized by human neutrophils but not internalized (Yamane et al., 2005; Yamanaka et al., 2009). Leid et al. (2002) have shown that human neutrophils can easily penetrate *S. aureus* biofilms but fail to phagocytose the bacteria. Similarly, in the murine model of systemic infection, the deletion of *ica* locus necessary for the biosynthesis of surface polysaccharide of *S. aureus* significantly reduces its virulence. A study in the early 1970s clearly showed that addition of the slime from *P. aeruginosa* cultures to *E. coli* or *S. aureus* dramatically inhibited phagocytosis by neutrophils (Schwarzmann and Boring III, 1971). In our previous study, we observed the restoration of the induction of abscess formation in mice when the purified EPS from the biofilm-forming strain of *P. nigrescens* was added to the cultures of a biofilm-non-forming mutant and injected into mice (Yamane et al., 2005). Though we have to carefully investigate the possibility that multiple mutations exist in EPS negative variants and lead to the observed incapability to induce abscesses in mice, it is conceivable that biofilm bacteria being held together by EPS might present a huge physical challenge for phagocytosing neutrophils. As a consequence of these neutrophils being frustrated by their inability to phagocytose this bacterial mass, this might trigger the unregulated release of bactericidal compounds that could cause tissue injury as shown in the inflammatory pathway associated with lung injury or chronic wounds (Moraes et al., 2006; Bjarnsholt et al., 2008). The cellular components from neutrophils themselves are known to exert a stimulatory effect on the developing *P. aeruginosa* biofilm when the host fails to eradicate the infection. We recently compared the level of pathogenicity on the clinical strains of *P. intermedia* with EPS productivity to those of several laboratory reference strains of periodontopathic bacteria (*P. intermedia* ATCC 25611, *P. gingivalis* ATCC 33277, *P. gingivalis* 381 and *P. gingivalis* W83; strains without producing polysaccharides as described above) in terms of the abscess formation in mice. EPS-producing *P. intermedia* strains 17 and OD1-16 induced abscess lesions in mice at 10^7 CFU, but other periodontopathic bacteria did not when tested at this cell concentration (Yamanaka et al. 2011). Resistance of *P. intermedia* with EPS productivity against the phagocytic activity of human neutrophils was stronger than those of *P. intermedia* ATCC 25611 and *P. gingivalis* ATCC 33277 that lack the capacity to produce polysaccharides (Fig. 5). Therefore, it is plausible that the antiphagocytic effect of EPS confers the ability to *P. intermedia* to induce abscess in mice at a small inoculation size.

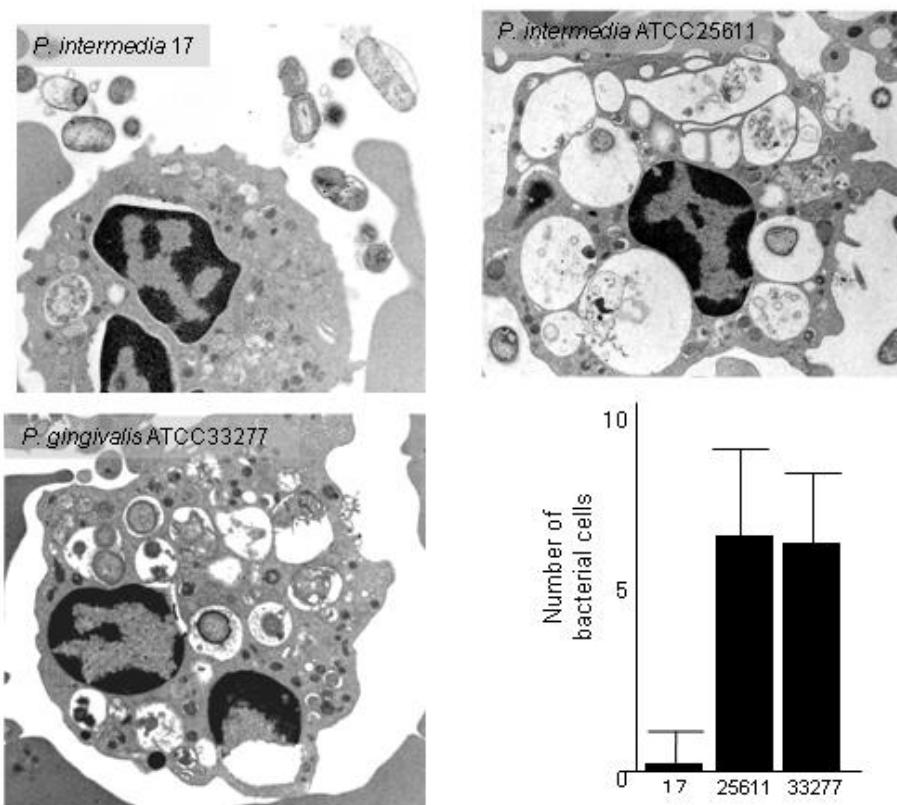


Fig. 5. Resistance of EPS-producing *P. intermedia* strain 17 against the phagocytic activity of human neutrophils. Test strains were co-cultured with human neutrophils for 90 min. Under transmission electron microscopy (TEM), 30 neutrophils were arbitrarily selected, and the number of bacterial cells engulfed in each cell was counted. Strain 17 cells were not engulfed by neutrophils. In contrast, *P. intermedia* ATCC 25611 and *P. gingivalis* ATCC 33277 cells were internalized and found within cytoplasmic vacuoles.

3. Conclusion

The matured dental plaque via the ordered sequence of events is undoubtedly a very important reservoir of periodontopathic pathogens. However, combined recent evidences together, it is plausible that initial colonizers including Gram-negative anaerobes can form biofilm by a self-synthesized matrix. If the initial colonizers assume an ability to produce EPS, this could contribute to the pathogenicity of the organisms by conferring their ability to evade the host's innate defense response. Some of the initial colonizers who have formed their own biofilm might be recognized by neutrophils in the gingival crevice but the neutrophils can not eradicate the bacterial cells due to the existence of EPS as the matrix of biofilm. This could be one of many etiologies of tissue injury found in chronic periodontitis lesions. Our hypothetical idea is described in Figure 6.

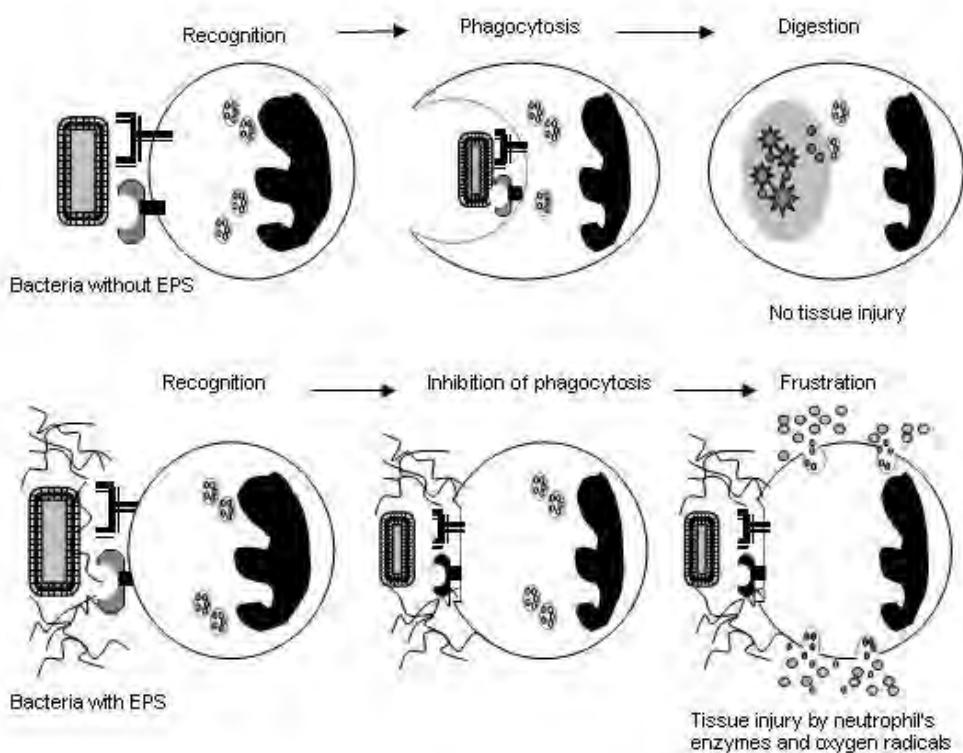


Fig. 6. Schematic depiction of tissue injury by neutrophils frustrated with unsuccessful phagocytosis of EPS-producing bacterial cells.

Finally, it is important to point out that many virulence phenotypes, especially the EPS productivity, expressed in natural environmental niches could be immediately lost through laboratory passages (Fux et al., 2005). Therefore, freshly isolated clinical strains are needed to re-evaluate the pathogenicity of periodontopathic bacteria isolated from the dental plaque or periodontal lesions.

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